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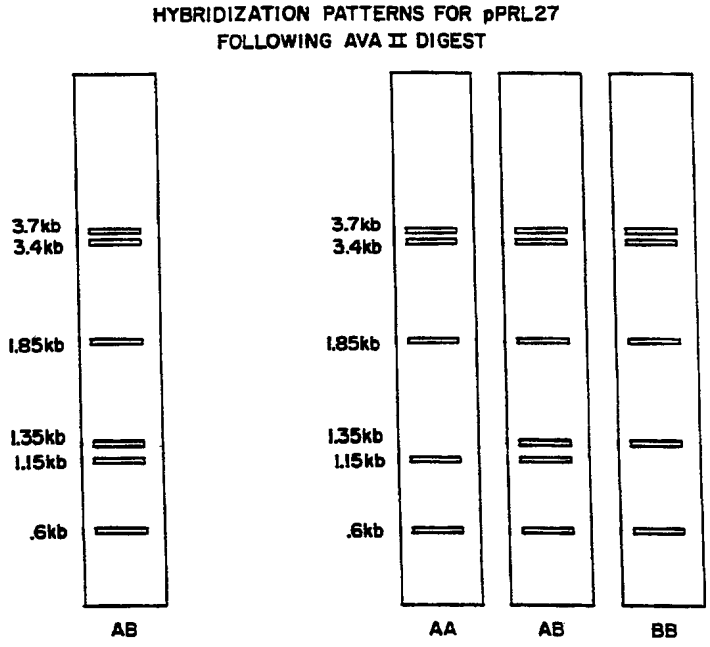
(54) Title: GENETIC MARKER FOR SUPERIOR MILK PRODUCTION IN DAIRY CATTLE



(57) Abstract

An assay for a genetic marker associated with increased milk production is disclosed. Also disclosed are kits for use in connection with the assay and breeding methods that use the assay. The assay centers on finding a genetic marker (e.g. at 1.15 kb) in a bovine cell (e.g. in the DNA of the cell). The presence of the marker is confirmed by exposing a gene sequence from the cell to a restriction enzyme so as to yield gene fragments of varying lengths. One then separates at least some of the fragments from others (such as by using electrophoresis), and one then hybridizes a plurality of probes that contain a portion of bovine prolactin sequence to the separated fragments. The probe is radio-labelled. One then compares the results (e.g. AA, Aa, or aa) of the hybridization with the hybridization results for a gene sequence known to either have the marker (e.g. Aa) or not have the marker (e.g. aa). The assay appears to be of greatest utility in connection with the Carlin-M Ivanhoe Bell Holstein family.

Genetic Marker For Superior Milk Production In Dairy Cattle

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Technical Field

The present invention relates to recombinant DNA technology. More specifically it relates to a means of determining from restriction fragment hybridization patterns whether a gene polymorphism associated with improved milk production is present in a bovine cell.

Background Art

With the competitive pressures that the dairy industry is facing, there has been significant interest in breeding and selecting dairy cattle which have improved milk production characteristics. Significant improvements have been achieved using standard breeding techniques in which progeny are studied. Their production results are then used to guide further breeding. One particularly successful family (from a milk production standpoint) is the Holstein line deriving from Carlin-M Ivanhoe Bell (registration number 1667366, Holstein-Friesian Association, Brattleboro, Vermont). It has been estimated that currently more than 25% of the highest total performance index Holstein bulls in the United States are progeny of this individual.

Unfortunately, such standard techniques require years to evaluate the true genetic value by progeny testing each bull. During progeny testing, many cows must be bred and give birth to offspring. The females must be raised, bred, allowed to give birth and, finally milked for a minimum length of time. The costs of confirming that a particular bull has superior genetics is therefore very high.

Given the problems involved in using standard selection techniques, some have tried to improve milk production by locating genes that express proteins important to milk production, cloning them, and then adding commercially produced amounts of these proteins to feeds, drugs, and the like. Various bovine genes have in fact been shown to express proteins that are important for the control of mammary growth, lactogenesis, and/or lactation. One of these, bovine prolactin, is approximately 10 kilobases (kb) in length. See S. Camper et al., 3 DNA 237-249 (1984). Unfortunately, there has been significant political and regulatory resistance to the introduction of such methods.

Various other research has discovered that polymorphisms (change in the genetic code) can be associated with recognizable differences in restriction fragment lengths of certain portions of the human genome. This has been of value in creating an assay for certain genetic diseases in humans. See e.g. D. Botstein et al., 32 Am. J. Human Gene. 314-331 (1980).

Polymorphisms which do not affect amino acid composition have been reported adjacent to the bovine prolactin gene. These bovine prolactin studies have generally focused on differences around these loci between breeds or among individuals of an undetermined relationship. To date, applicants are unaware of anyone else having successfully located any polymorphism associated with a bovine gene which is indicative of improved milk production.

Thus, it can be seen that a need exists for a means of more efficiently selecting and breeding cattle for the trait of improved milk production.

Disclosure Of Invention

In one embodiment, there is provided an assay for the presence in a bovine gene sequence of a genetic marker that is located within 1.5 kb of a bovine prolactin coding exon in the sequence. The marker is indicative of an inheritable trait of increased milk production in progeny.

The assay involves exposing the gene sequence to a restriction enzyme (e.g. Ava II) so as to yield gene fragments of varying lengths; then separating at least some of the fragments from others (e.g. using electrophoresis); then hybridizing a plurality of probes (e.g. radio-labelled cDNA probes) that contain a portion of a bovine prolactin gene sequence to the separated fragments; and then comparing the results of the hybridization with assay results for a bovine gene sequence known to have the marker or a bovine gene sequence known not to have the marker. The preferred bovine gene sequence is from a Holstein Carlin-M Ivanhoe Bell cell or its progeny.

In another embodiment, the invention provides a kit for assaying for the presence in a bovine gene sequence of a genetic marker that is located within 1.5 kb of a bovine prolactin coding exon in the sequence, the marker being indicative of an inheritable trait of increased milk production in progeny. The kit has a probe containing a portion of a bovine prolactin gene sequence, and also a bovine gene sequence known to contain said marker. The probe is preferably a cDNA sequence of a portion of bovine prolactin and the probe can be radio-labelled.

The gene sequence containing the marker is preferably a sequence contained in the cell of ATCC 40573, or its progeny, or sequences derived from either. The kit may also contain a restriction enzyme such as Ava II.

In another embodiment there is a breeding method whereby one conducts an assay of the above type on a plurality of gene sequences from different bovine cells to be selected from, and one then drops out of the breeding program at least one of the cells (or its progeny) that do not contain the marker.

It will be appreciated that the present invention can reduce the number of animals selected to achieve the same goal and reduce breeding costs:

1. Young bull calves can be tested before entry into sire programs. Those without the marker would be selected not to be continued in the program.

2. Daughters of bulls in this family who are being considered as mates could be tested. Those that are of an especially elite type AA (as described below) could be selected as preferable because they increase the chances for the elite marker being passed along.

3. When the line goes to the commercial stage, daughters could be tested at birth. Those not having the marker could be culled, and those having it could be used for milk production.

4. The screening process could be used to lower the number of bulls needed to be tested to maintain the same selection advantages as exist today.

It should be appreciated that the marker gene provides information as a supplement to other traditional tools for selection. However, in cases of equal pedigree merit, the marker will help distinguish the lines, and thus lead to substantial improvements at much lower cost, and much more quickly. In the analyses conducted thus far, it appears that the marker, all other things being equal, is associated with a significant improvement in milk production in the Carlin-M Ivanhoe Bell family.

Thus, the objects of the present invention include:

(a) providing an assay of the above kind for the presence of a genetic marker associated with improved milk production traits;

(b) providing a kit of the above kind to be used in connection with such assays;

(c) providing a breeding method of the above kind for using such assays;

(d) producing cattle by using breeding methods of the above kind; and

(e) providing such assays, kits, and methods so as to save time and money.

These and still other objects and advantages of the present invention will be apparent from the description which follows. In this description, the preferred embodiments of the invention will be described with reference to the accompanying drawings. These embodiments do not represent the full scope of the invention. Rather, the invention may be employed in other embodiments. Reference should therefore be made to the claims to interpret the breadth of the invention.

Brief Description Of The Drawings

Fig. 1 depicts (in schematic form) hybridization patterns of a heterozygus sire and of three possible sons.

Best Modes For Carrying Out The Invention

We followed the following general steps:

1. Extraction Of DNA: Semen from commercially available Holstein bulls (or other bovine cells) provided the source of the DNA to be tested. Spermatozoa were then treated so that their DNA would be released from the cell and concentrated in relatively pure form.

2. Digestion And Fragment Separation: A restriction enzyme (preferably Ava II) which recognizes a sequence in double stranded DNA near bovine prolactin was used to cleave the DNA. DNA fragments were separated by electrophoresis in agarose gels against standards of known size. The gels were stained with ethidium bromide and photographed. Fragments of DNA were transferred to nylon membranes.

3. Hybridization: Blots were hybridized in high stringency conditions to bovine prolactin cDNA that had been radio-labelled by nick-translation. Blots were washed with solutions of decreasing salt concentration to remove any nonspecifically bound probe. Blots bound to the labelled probe were exposed to autoradiography film with intensifying screens for three to five days at low temperature.

4. Correlation To Data On Milk Production Of Progeny: Three genotypes were discovered by analysis of hybridization patterns of a particular family of sons.

1. Extraction

Semen from commercially available registered U.S. Holstein progeny bulls of the Carlin-M Ivanhoe Bell family provided the source material from which genomic DNA was isolated. DNA was extracted from sperm using a procedure modified from E. Borenfreund et al., 297 Nature 1375-77 (1961). Briefly, frozen .5 ml artificial insemination units containing approximately 30 x 10^6 sperm/unit were allowed to thaw at room temperature. The thawed semen was then treated with 2-mercaptoethanol in 10 mM Tris, pH 8.0, 100 mM NaCl, 50 mM EDTA and 0.25% sodium dodecyl sulfate (SDS) and incubated at 53° to 55°C for 30 minutes. Semen samples were cooled in an ice bath for 10 minutes prior to addition of proteinase K to a final concentration of 200 ug/ml and incubation continued at 37°C for 3 hours. DNA was extracted sequentially with phenol followed by phenol:chloroform:

isoamyl alcohol (25:24:1) and finally chloroform:isoamyl alcohol (24:1).

Following extraction, the DNA was precipitated with an equal volume of cold isopropanol (-20°C). The DNA was removed by spooling it onto a polyethelene pipet tip and air dried before being dissolved in 10 mM Tris, pH 7.5, containing 1 mM EDTA and 50 mM NaCl.

Concentrations of DNA in each sample were estimated by their optical density at 260 nm. Samples were stored at 4°C.

2. Digestion/Separation

Prior to enzymatic digestion of the DNA, 15 µg of isolated genomic DNA were dialyzed against a buffer compatible with the restriction endonuclease employed. The preferred restriction enzyme to digest the sperm genomic DNA from subsets of bulls was Ava II (New England BioLabs, Beverly, Massachusetts). DNA samples were digested in accordance with the enzyme manufacturer's standard recommendations for at least six hours.

The resulting DNA fragments were separated by electrophoresis in agarose gels (1.0% agarose) in 40 mM Tris, 20 mM NaCl, 20 mM acetic acid and 2 mM EDTA. After completion of electrophoresis, gels were stained with ethidium bromide (2 µg/ml) and photographed using UV light. Transfer to DNA to Hybond-N membrane (Amersham, Arlington Heights, Illinois) was accomplished using the manufacturer's recommended modified Southern blotting method. See generally E.M. Southern, 98 J. Mol. Biol. 503-517 (1975).

DNA fragments were crosslinked to the nylon membrane by baking for 2 hours at 80°C followed by a two minute exposure to 300 NM UV light from a transilluminator (Fotodyne, New Berlin, Wisconsin).

3. Hybridization

Blots were prehubridized on the membrane in 5x SSPE (0.9 M NaCl, 25 nM sodium phosphate, pH 7.4 and 2.5 mM EDTA) 0.4% SDS, 50% deionized formamide, 5x Denhardts (0.1% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), and denatured herring sperm (50µg/ml) at 42°C for 6 hours.

A plasmid containing a portion of bovine prolactin cDNA (pBPRL27) was radio-labelled by nick-translation. See e.g. P. Rigby et al., 113 J. Mol Biol. 237-251 (1977). pBPRL27 is deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A. as ATCC No. 40574 on February 15, 1989. Samples from the deposit are available in accordance with U.S. patent law requirements upon issuance of the patent and the requirements of any applicable foreign patent laws. No patent license is intended by such availability. Another plasmid cDNA which could be used for this purpose is (pBPRL72) from N. L. Sasavage, et al. 257 J. Biol. Chem. 678-681 (1982).

The labelled probe was added with fresh hybridization solution and the incubation continued for 36 hours. Blots were washed twice with 2x SSC (300 mM NaCl and 30 mM Na citrate, pH 7.0) at 65°C for 15 minutes, followed by 2x SSC and 0.1% SDS at 65°C for 30 minutes. The final wash was at high stringency (0.1x SSC at 65°C for 10 minutes). Blots were exposed to Kodak XAR-5 film with intensifying screens for 3 to 5 days at -80°C. The probe was removed according to the manufacturer's recommendations for multiple probing of blots.

4. Correlation Analysis

The resulting hybridization patterns were analyzed. Three types were identified. The patterns of these types (labelled AA, AB, and BB, respectively) are shown in Figure 1 as possible offspring of type AB. Analysis of family lines shows that sons of the AA type all carry the A

allele, and sons of the BB type are certain of not carrying the marker. Sons of the AB type would be a mixture of those carrying either the B from the sire or A from the sire.

A statistical model was then formulated to test for differences in predicted genetic values for milk production traits between those carrying A from the sire versus those carrying B from the sire. Results of the analysis revealed a statistically significant higher genetic transmitting value for milk yield from the sons in this family who carried the A marker.

Figure 1 shows that the "most preferred" AA pattern shows fragments of about 1.15 kb, but not one at about 1.35. The second most preferred AB pattern has lines at 1.35 and 1.15. The 1.15 fragment is missing in the third (undesired) pattern.

Applicant has deposit a bovine sperm cell 14H9689 of type AB with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A., as ATCC No. 40573, on February 15, 1989. Samples from the deposit are available in accordance with U.S. patent law requirements upon issuance of the patent and the requirements of any applicable foreign patent laws. No patent license is intended by such availability. It will be appreciated that one skilled in the art can use this "known" to confirm the location of the key fragments' hybridization pattern.

Other Variants

It should be understood that the above description deals with a preferred embodiment of the invention, and that many other embodiments are within the scope of the invention. For example, the invention should work with other types of bovine cells that contain DNA (other than just sperm). In this regard, it should be applicable to other cells types.

Also, while Ava II restriction fragments associated with prolactin have been chosen as a model system, other

restriction enzymes when used with prolactin (or prolactin adjacent) probes may also yield characteristic hybridization patterns, that can be compared to knowns developed using the Ava II patterns. Moreover, while the primary utility of the invention is for Carlin-M Ivanhoe Bell progeny, the principles of the invention may also apply to other Holstein families.

Also, it should be noted that the presence of the marker is a statistical indication of improved production. Thus, breeders will also want to continue to use their standard breeding techniques when this marker is used. This marker does not replace such techniques. It supplements them.

Industrial Applicabilities

The present invention provides a means in industry to test the potential of various bulls and cows, and thereby render breeding techniques much more efficient.

Claims

We claim:

1. An assay for the presence in a bovine gene sequence of a genetic marker that is located within 1.5 kb of a bovine prolactin coding exon in the sequence, said marker being indicative of an inheritable trait of increased milk production in female progeny, said assay comprising:

(a) exposing the gene sequence to a restriction enzyme so as to yield gene fragments of varying lengths;

(b) separating at least some of the fragments from others;

(c) hybridizing a plurality of probes that contain a portion of a bovine prolactin gene sequence to the separated fragments; and

(d) then comparing the results of the hybridization with hybridization assay results for a bovine gene sequence known to have the marker or for a bovine gene sequence known not to have the marker.

2. The assay of claim 1, wherein the bovine gene sequence is from a Holstein bovine cell.

3. The assay of claim 2, wherein the bovine gene sequence is from a Carlin-M Ivanhoe Bell cell, or its progeny, or a cell derived from either.

4. The assay of claim 3, wherein the restriction enzyme is Ava II, and the probe is a labelled cDNA probe.

5. The assay of claim 4, wherein the fragments are separated by electrophoresis.

6. A kit for assaying for the presence in a bovine gene sequence of a genetic marker that is located within 1.5 kb of a bovine prolactin coding exon in the sequence, the marker being indicative of an inheritable trait of increased milk production in progeny, said kit comprising:

a probe containing a portion of a bovine prolactin gene sequence; and

a bovine gene sequence known to have the marker.

7. The kit of claim 6, wherein the probe is a cDNA sequence of a portion of bovine prolactin.

8. The kit of claim 7, wherein the probe is a radio-labelled cDNA of bovine prolactin.

9. The kit of claim 6, wherein the bovine gene sequence known to have the marker is from ATCC 40573, or its progeny, or gene sequences derived from either.

10. The kit of claim 6, wherein the kit further comprises the restriction enzyme Ava II.

11. A breeding method comprising the steps of:

conducting the assay of claim 1 on a plurality of bovine gene sequences from different bovine cells; and

then selecting out at least one of the cells whose gene sequence did not contain the marker.

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HYBRIDIZATION PATTERNS FOR pPRL27
FOLLOWING AVA II DIGEST

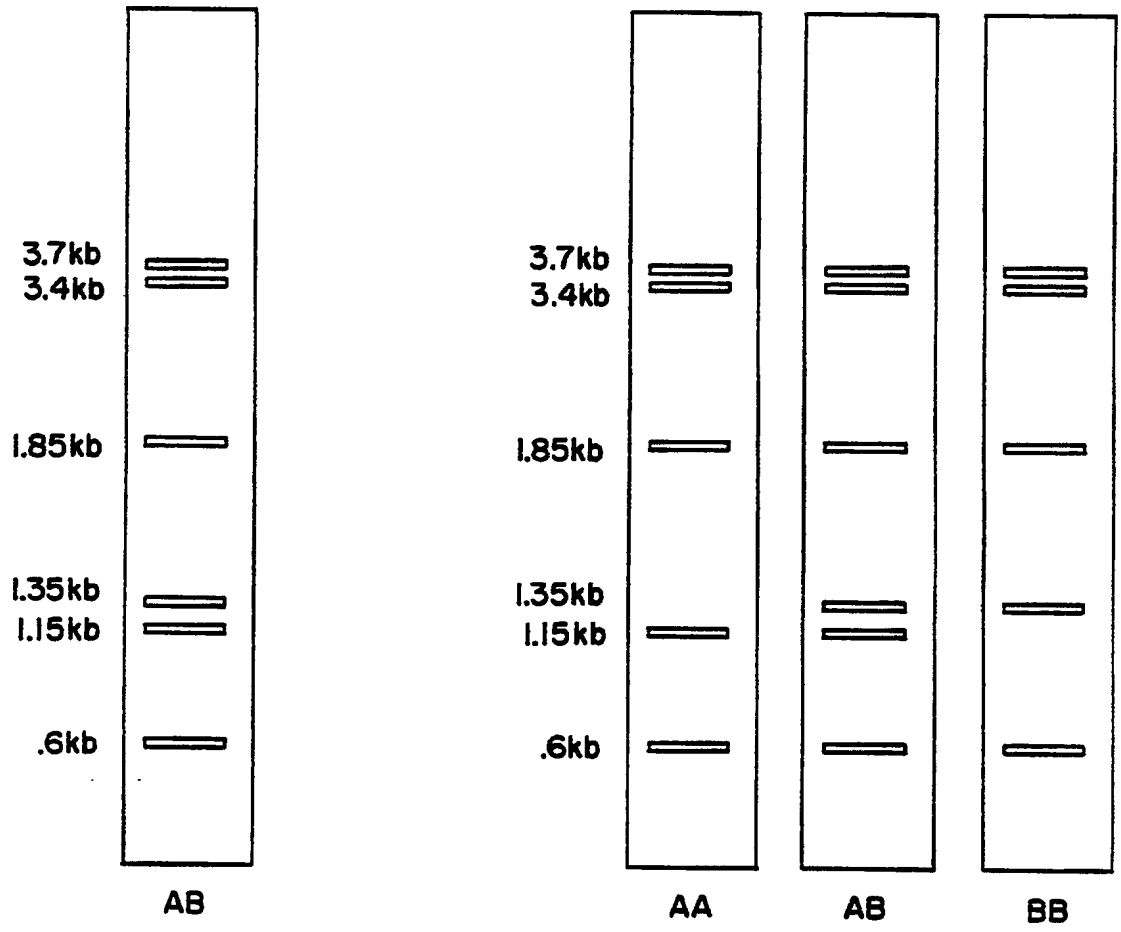

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/00932

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	Dialog Information Services, File 55, Biosis 81-90, BIOSIS number 88061287, Cowan et al: "Restriction fragment length polymorphisms associated with growth hormone and prolactin genes in holstein bulls evidence for a novel growth hormone allele", Anim Genet 20 (2), 1989, 157-166	1-2,6-8, 11
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X	Dialog Information Services, File 55, Biosis 81-90, BIOSIS number 87078218, Hallerman E M et al: "Screening of israeli holstein-friesian cattle for restriction fragment length polymorphisms using homologous and heterologous DNA probes", J Dairy Sci, 71 (12), 1988, 3378-3389	1-2,5-8, 11
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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd May 1990	- 5. 06. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Dialog Information Services, File 55, Biosis 81-90, BIOSIS number 85117779, Prokop C-M et al: "Description of restriction fragment length polymorphisms for the milk protein genes in cattle and their possible application in animal breeding", J Anim Breed Genet 105 (1), 1988, 70-80	1-2,6-8, 11
X	Dialog Information Services, File 55, Biosis 81-90, BIOSIS number 85015606, Hallerman E M et al: "Restriction fragment length polymorphisms in dairy and beef cattle at the growth hormone and prolactin loci", Anim Genet 18 (3), 1987, 213-222	1-2,6-8, 11
X	Dialog Information Services, File 55, Biosis 81-90, BIOSIS number 78091432, Camper S A et al: "Characterization of the bovine prolactin gene", DNA (N Y) 3 (3), 1984, 237-250	1,6-8, 11
A	Dialog Information Services, File 55, Biosis 81-90, BIOSIS number 87023011, Klindt J: "Relationships among growth hormone and prolactin secretory parameter estimates in holstein bulls and their predicted differences for lactational traits", J Anim Sci 66 (11), 1988, 2784-2790	1-11
A	WO, A2, 8809386 (BOURLET DE LA VALLEE) 1 December 1988, see the whole document	1-11
A	EP, A1, 0237362 (CETUS CORPORATION) 16 September 1987, see the whole document	1-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/00932

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 07/05/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A2- 8809386	01/12/88	FR-A-B- 2615865	02/12/88
EP-A1- 0237362	16/09/87	AU-D- 6996287	17/09/87
		JP-A- 62214355	21/09/87

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

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